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## **Administration of the hyper-immune bovine colostrum extract IMM-124E ameliorates experimental murine colitis**

Spalinger, Marianne R ; Atrott, Kirstin ; Baebler, Katharina ; Schwarzfischer, Marlene ; Melhem, Hassan ; Peres, Dan R ; Lalazar, Gadi ; Rogler, Gerhard ; Scharl, Michael ; Frey-Wagner, Isabelle

**Abstract:** Background/Aims Inflammatory bowel disease (IBD) is accompanied by lesions in the epithelial barrier, which allow translocation of bacterial products from the gut lumen to the host's circulation. IMM-124E is a colostrum-based product, containing high levels of anti-E.coli-LPS IgG and might limit exposure to bacterial endotoxins. Here, we investigated whether IMM-124E can ameliorate intestinal inflammation. Methods Acute colitis was induced in WT C57Bl/6J mice by administration of 2.5% DSS for 7 days. T cell transfer colitis was induced via transfer of  $0.5 \times 10^6$  naïve T cells into RAG2 -/- C57Bl/6J mice. IMM-124E was administered daily by oral gavage either preventive or therapeutically. Results Treatment with IMM-124E significantly ameliorated colitis in acute DSS colitis and in T cell transfer colitis. Maximum anti-inflammatory effects were detected at an IMM-124E concentration of 100 mg/kg body weight, while 25 mg/kg and 500 mg/kg were less effective. Histology revealed reduced levels of infiltrating immune cells, and less pronounced mucosal damage. Flow cytometry revealed reduced numbers of effector T helper cells in the intestine, while levels of regulatory T cells were enhanced. IMM-124E-treatment reduced the DSS-induced increase of serum levels of LPS-binding protein, indicating reduced systemic LPS exposure. Conclusions Our results demonstrate that oral treatment with IMM-124E significantly reduces intestinal inflammation, via decreasing the accumulation of pathogenic T cells, and concomitantly increasing the induction of regulatory T cells. Our study confirms the therapeutic efficacy of IMM-124E in acute colitis and suggests that administration of IMM-124E might represent a novel therapeutic strategy to induce or maintain remission in chronic colitis.

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1   **Administration of the hyper-immune bovine colostrum extract IMM-**  
2                   **124E ameliorates experimental murine colitis**

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19   Australia.

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21   **Short title:** hyper-immune bovine colostrum ameliorates colitis

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## 24    **Abstract**

25    **Background/Aims:** Inflammatory bowel disease (IBD) is accompanied by lesions in  
26    the epithelial barrier, which allow translocation of bacterial products from the gut  
27    lumen to the host's circulation. IMM-124E is a colostrum-based product, containing  
28    high levels of anti-E.coli-LPS IgG and might limit exposure to bacterial endotoxins.  
29    Here, we investigated whether IMM-124E can ameliorate intestinal inflammation.

30    **Methods:** Acute colitis was induced in WT C57Bl/6J mice by administration of 2.5%  
31    DSS for 7 days. T cell transfer colitis was induced via transfer of  $0.5 \times 10^6$  naïve T  
32    cells into RAG2<sup>-/-</sup> C57Bl/6J mice. IMM-124E was administered daily by oral gavage  
33    either preventive or therapeutically.

34    **Results:** Treatment with IMM-124E significantly ameliorated colitis in acute DSS  
35    colitis and in T cell transfer colitis. Maximum anti-inflammatory effects were  
36    detected at an IMM-124E concentration of 100 mg/kg body weight, while 25 mg/kg  
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38    immune cells, and less pronounced mucosal damage. Flow cytometry revealed  
39    reduced numbers of effector T helper cells in the intestine, while levels of regulatory  
40    T cells were enhanced. IMM-124E-treatment reduced the DSS-induced increase of  
41    serum levels of LPS-binding protein, indicating reduced systemic LPS exposure.

42    **Conclusions:** Our results demonstrate that oral treatment with IMM-124E  
43    significantly reduces intestinal inflammation, via decreasing the accumulation of  
44    pathogenic T cells, and concomitantly increasing the induction of regulatory T cells.  
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46    suggests that administration of IMM-124E might represent a novel therapeutic  
47    strategy to induce or maintain remission in chronic colitis.

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49 **Keywords:** systemic LPS exposure, novel therapeutic approach in IBD,

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## 51 **Introduction**

52 Inflammatory bowel disease (IBD), with its two main sub-forms ulcerative colitis  
 53 (UC) and Crohn's disease (CD), is an important health problem with worldwide  
 54 increasing prevalence<sup>1, 2</sup>. IBD is characterized by chronic and relapsing intestinal  
 55 inflammation and affected patients suffer from significant clinical symptoms such as  
 56 abdominal pain, diarrhoea or fever<sup>3</sup>. Extra-intestinal manifestations such as  
 57 inflammation of the skin, joints, liver or the eyes are common complications<sup>4, 5</sup>.  
 58 Though the exact pathophysiology is still unknown, it is evident that genetic factors,  
 59 environmental triggers, and aberrant immune responses towards the commensal  
 60 microbiota contribute to the development of IBD<sup>2, 5-7</sup>.

61  
 62 The human intestine is populated by up to  $10^{13}$  microbes, which form a complex  
 63 ecosystem<sup>8, 9</sup>. While these microbes are usually harmless or even beneficial to the  
 64 host<sup>10</sup>, some microbial products, including the bacterial cell wall component  
 65 lipopolysaccharide (LPS), can promote immune responses and thereby contribute to  
 66 the development, progression, and exacerbation of IBD<sup>11</sup>. To prevent translocation of  
 67 pro-inflammatory products from the gut lumen to the systemic circulation, where they  
 68 elicit strong immune reactions, an adequate protective immunity towards invading  
 69 bacteria is crucial. This includes a tight epithelial barrier, as well as rapid clearance of  
 70 bacterial products and invading pathogens by innate immune cells located directly  
 71 beneath the epithelial layer<sup>9, 12</sup>. In addition, the production of immunoglobulins (Ig)  
 72 against bacterial products prevents those molecules to pass the epithelial surface<sup>13, 14</sup>.  
 73 Further, IgA seems to be involved in determining the composition of the intestinal  
 74 microbiome<sup>13, 14</sup>, which is also a crucial factor for intestinal health<sup>8</sup>. In IBD, these first  
 75 line defence mechanisms are disturbed, and increased levels of bacterial products,

including LPS are detected in the serum<sup>15-17</sup>. LPS triggers the activation of toll-like receptor (TLR)4 on host cells, resulting in production of IL-6, tumour necrosis factor (TNF), interferon (IFN)- $\gamma$ , and nitric oxide, which contributes to pro-inflammatory reactions and exacerbation of the disease<sup>15</sup>.

Colostrum is the milk produced by lactating mammals within the first 72 h after giving birth. In comparison to normal milk, colostrum contains high levels of immune-active molecules, such as IgA/IgG, growth factors, anti-microbial agents (e.g. lysozyme and lactoperoxidase), as well as lactoferrin and vitamins<sup>18-20</sup>. In contrast to human colostrum, which is rich in IgA, the main immunoglobulin found in bovine colostrum is IgG. This is of interest, since IgA can pass mucosal surfaces via the IgA transporter and possibly translocate to the systemic circulation, while IgG remains in the intestinal lumen. IMM-124E is a bovine colostrum-based product obtained from cows that have been immunized with LPS from *Escherichia coli*, which results in high levels of LPS-specific IgG. A study investigating the composition of IMM-124E has been published recently, showing that 1mg IMM-124E powder contains about 0.4mg total IgG, which is comparable to the total IgG content in colostrum powder<sup>21</sup>. In previous studies, it has been demonstrated that administration of IMM-124E ameliorates disease in a model of non-alcoholic steatohepatitis (NASH)<sup>22, 23</sup>, a disease where chronic presence of LPS and subsequent low-grade inflammation might promote disease activity<sup>24, 25</sup>. Further, in TNBS-induced colitis, IMM-124E treatment resulted in reduced colitis severity and promoted the development of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells<sup>26</sup>, but its effect on barrier-disruption-mediated colitis has not been addressed so far. In addition, IMM-124E treatment fostered the development of CD25<sup>high</sup> regulatory T helper and

101 regulatory NKT cells in a mouse model of NASH<sup>22</sup>, indicating that IMM-124E might  
102 have potent immune cell modulatory effects.  
103 Here, we further investigated the anti-inflammatory potential of IMM-124E in two  
104 mouse models of colitis, namely the dextran sodium sulphate (DSS) model of acute  
105 barrier disruption-induced colitis and the naïve T cell transfer model of colitis, to  
106 provide the rationale for its use in IBD patients.

## 107 **Methods**

108 **Mice, colitis induction, weight recording, and IMM-124E treatment.** All animal  
 109 experiments were conducted according to Swiss animal welfare legislation and were  
 110 approved by the local animal welfare office (Cantonal veterinary office Zurich;  
 111 licence number ZH121/2017). Female C57Bl/6J mice in a weight range between 21  
 112 and 23 g were obtained from Janvier Elevages (France) and maintained in a specific  
 113 pathogen free (SPF) environment with water and food *ad libitum*. To induce acute  
 114 DSS colitis, mice were randomized into groups with equal average weight and  
 115 administered 2.5% dextran sodium sulphate (DSS, MW 36'000-50-000) in the  
 116 drinking water for 7 days. RAG2<sup>-/-</sup> mice on a C57Bl/6J background were initially  
 117 obtained from Taconic (Rensselaer, NY), and a local colony maintained in our SPF  
 118 facility. To induce T cell transfer colitis, CD4<sup>+</sup>CD62L<sup>high</sup>CD44<sup>low</sup> naïve T cells were  
 119 sorted from the spleen of C57Bl/6J donor mice and 0.5x10<sup>6</sup> T cells injected per  
 120 RAG2<sup>-/-</sup> recipient. After 4 weeks, mouse endoscopy was performed as described  
 121 below, and mice randomized into groups with equal colitis severity prior to start of  
 122 treatment. Weight of the mice was recorded daily at the same time. At the last day of  
 123 the experiment, colonoscopy (see below) was performed in all animals. Directly after  
 124 colonoscopy, blood was collected to obtain serum and mice were sacrificed via  
 125 cervical dislocation prior to tissue collection for downstream applications. IMM-124E  
 126 and colostrum control powder was provided by Immuron Ltd. (Melbourne, Australia)  
 127 and freshly emulsified at an appropriate concentration in autoclaved water directly  
 128 prior to administration via oral gavage. The following treatment groups were used in  
 129 DSS colitis: preventive treatment: 100 mg/kg body weight or 500 mg/kg IMM-124E  
 130 per day, starting two days before beginning of DSS application (day 2 of the  
 131 experiment). Administration of 100 mg/kg colostrum from non-immunized cows



served as a treatment control. Therapeutic treatment: 500 mg/kg IMM-124E per day, starting at day three of the DSS treatment. In transfer colitis, the following treatment groups were studied: 25 mg/kg, 100 mg/kg, or 500 mg/kg IMM-124E once per day. Administration of 100 mg/kg BSA or transfer of regulatory T cells served as controls.

**Mouse endoscopy.** To assess macroscopic colitis severity, mice were anesthetised i.p. with 100 mg/kg body weight ketamine (Vétoquinol, Bern, Switzerland) and 8 mg/kg body weight Xylazine (Bayer, Lyssach, Switzerland). Animals were examined as described previously<sup>27</sup>: recording was performed with the Karl Storz Tele Pack Pal 20043020 (Karl Storz Endoskope, Tuttlingen, Germany), and colitis severity scored using the murine endoscopic index of colitis severity (MEICS) scoring system as described previously<sup>28</sup> using the following five parameters: (1) transparency of the colon, (2) changes of the vascular pattern, (3) fibrin visible, (4) granularity of the mucosal surface and (5) stool consistency.

**Colon length measurement.** The entire colon was resected from each mouse and the length from the cecum to the anus measured prior to dissection to obtain tissue pieces for downstream applications such as histology, RNA/protein isolation, and MPO assay.

**Histology.** For histological assessment of colitis severity, mice were sacrificed and 1.5cm long pieces from the terminal colon collected and transferred into 4% formalin solution. After 24h, pieces were dehydrated in ascending alcohol series and HistoClear® (Chemie Brunschwig, Basel, Switzerland) prior to embedding in paraffin. The samples were cut into 4µm thin sections, transferred onto glass cover

slides and air-dried. Sections were then rehydrated (incubation in Histoclear®, followed by descending ethanol series), incubated for 10 min. in Hemalaun solution (Chemie Brunschwig), rinsed for 10 min. in H<sub>2</sub>O, incubated for 15 sec. in Eosin solution (2% w/v; Sigma-Aldrich, Buchs Switzerland), briefly rinsed in H<sub>2</sub>O, dehydrated in ascending alcohol series and Histoclear, and finally mounted with Histopaque embedding solution (Chemie Brunschwig). The extent of colitis was assessed using the following parameters as described previously<sup>29</sup>: epithelial damage (0= normal appearance, 1=partial loss of goblet cells, 2=extended loss of goblet cell, 3=loss of crypt in small areas, 4=extended loss of crypts) and infiltration/inflammation (0=no infiltration or thickening, 1=infiltration around the crypt area, 2=infiltration of the mucosa, 3=infiltration of the submucosa, mild oedema, 4=infiltration reaches the *muscularis submucosae*, pronounced oedema).

**Myeloperoxidase (MPO) activity assay.** Colon specimens were homogenized in 50 mM phosphate buffer (pH 6.0) and 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich) using a gentleMACS tissue homogenizer (Miltenyi Biotec, Bergisch Gladbach, Germany). After three freeze and thaw cycles, supernatant was mixed with 0.02% dianisidine (Sigma-Aldrich) in 50 mM phosphate buffer, pH 6.0, and 0.0005% H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich). Myeloperoxidase activity, expressed as arbitrary units, was calculated as mean absorbance (460 nm) per incubation time (in min) per protein content (in g).

**RNA extraction and RT-PCR.** Colon tissue was mechanically dissociated and lysed in RLT buffer (Qiagen, Venlo, Netherlands) using a GentleMACS tissue homogenizer (Miltenyi Biotec). Total RNA was isolated from the lysates using RNeasy Mini Kit

(Qiagen), and DNA removed by TURBO DNA-free Kit (Ambion, Austin, TX) according to manufacturer's instructions. RNA concentration was assessed by absorbance at 260 and 280 nm. Complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Real-time PCR was performed using FAST qPCR MasterMix for Taqman Assays (Applied Biosystems) on a Fast HT7900 Real-Time PCR system using SDS Software (Applied Biosystems) or on a QuantStudio 6 System from Thermo Fisher Scientific (Waltham, MA). Measurements were performed in triplicates, mouse GAPDH was used as endogenous control, and results were analyzed by the  $\Delta\Delta CT$  method. The real-time PCR contained an initial enzyme activation step (5 min, 95 °C) followed by 45 cycles consisting of a denaturing (95 °C, 15 sec) and an annealing/extending (60 °C, 1 min) step. The used gene expression assays were all obtained from Thermo Fisher Scientific.

**Protein extraction and Western blot.** Colon pieces were disrupted in M-PER buffer (Thermo fisher Scientific) using a GentleMACS tissue homogenizer. Equal amounts of proteins were loaded on polyacrylamide gels and after separation by gel-electrophoresis blotted onto nitrocellulose membranes. Membranes were blocked in a 1% BSA and 3% Milk containing blocking solution, and incubated over night in blocking solution with an appropriate concentration of primary antibody. Membranes were washed three times with washing buffer (Tris buffered saline containing 1% Tween 20 (1%TBST)) before incubation with HRP-coupled anti-rabbit secondary antibody (Lab Force, Santa Cruz, CA) for 2 hours. Immunoreactive proteins were detected with a Fusion Solo S imager

(Vilber Lourmat, Witec AG, Littau, Switzerland) using a Western Blotting detection kit (Western Bright Sirius or ECL, Advansta, Menlo Park, CA). Anti-phospho NF- $\kappa$ B-p65 (Ser536) and anti-NF- $\kappa$ B-p65, antibodies were obtained from Cell Signaling Technologies (Danvers, MA).

**IgG depletion from IMM-124E.** To deplete IgG from IMM-124E, the IMM-124E suspension was incubated with Sepharose A beads (250  $\mu$ l beads/ml suspension) for 2 h at room temperature. As control, IMM-124E suspension was incubated with non-protein coupled Sepharose beads for 2 h. The suspensions were then centrifuged at 250 x g for 2 min. and supernatants used for gavages.

**Colon Explants.** For colon explants, 1 cm of the colon was opened longitudinally, rinsed with PBS and cultured for 24 h in 0.5 ml RPMI (Thermo Fisher Scientific) supplemented with 10% FCS, and 1% Penicillin/Streptomycin solution (Thermo Fisher Scientific) in an incubator (37°C, 10% CO<sub>2</sub>).

**Enzyme-linked immunosorbent Assay (ELISA).** Cytokine measurement were performed using commercial ELISA kits from R&D (Minneapolis, MN; IL-6, IL-10, TNF, IFN- $\gamma$ ) and HycultBiotech (Uden, Netherlands; LBP ELISA). All Assays were performed according to the manufacturer's instructions.

**Flow cytometry.** Lamina propria immune cells were isolated as described previously<sup>27, 30</sup>. In brief, the colon was opened, rinsed in PBS, cut in 0.5cm pieces and epithelial cells removed by repeated incubation in EDTA-containing HBSS and rigorous shaking. Tissue pieces were then digested for 15-25 min. in RPMI

supplemented with 10% FCS, 0.6 mg/ml collagenase IV (Roche, Basel, Switzerland) and 2.4 mg/ml dispase (Sigma-Aldrich). Remaining tissue was homogenized using a 26G needle and passed over a 40um mesh. For intracellular cytokine staining, cells were treated with PMA (50 ng/ml, Sigma-Aldrich, Buchs, Switzerland), Ionomycin (1µg/ml, Sigma-Aldrich) in the presence of 10 ng/ml Brefeldin A (Sigma-Aldrich) for 3 h. Cells were harvested, washed in PBS, stained for surface molecules for 30 min., washed in FACS buffer (PBS, 2% FCS), and fixed in fixation/permeabilization buffer (BD, Franklin Lakes, NJ) for 15 min. Cells were washed and resuspended in permeabilization buffer (BD) prior to addition of anti-IFN-γ and anti-IL-17 antibody for 15 min. Before analysis, cells were washed in permeabilization buffer and resuspended in FACS buffer. A LSR Fortessa analyzer from BD was used for all analysis.

**LPS measurement in mouse serum.** HEKblue-mTLR4 cells (Invivogen) were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FCS and 2mM L-glutamine. For LPS detection, 20ul serum was added to 96 well plates and 200'000 HEKblue-mTLR4 cells in 180ul HEK-Blue detection medium (Invivogen) were added per well. After 24 h, absorbance at 630 nm was measured and concentrations calculated based on a standard curve prepared from pure LPS diluted in mouse serum.

**Statistics.** Unless otherwise stated, data are presented as means ± standard error of the mean (SEM) for one out of two independent experiments. n equals the number of mice in one experiment. Statistical analysis was performed by analysis of variance

256 (ANOVA) followed by Mann-Whitney U and Bonferroni correction was used to  
257 correct for multiple testing. P values  $< 0.05$  were considered significant.

## Results

### Administration of IMM-124E prevents acute DSS-induced colitis.

To test whether administration of IMM-124E is able to ameliorate colitis *in vivo*, we induced acute colitis in WT C57Bl/6J mice via administration of 2.5% DSS in the drinking water for 7 days. This treatment resulted in severe intestinal inflammation, characterized by diarrhoea, severe weight loss (Figure 1A), macroscopic signs of colitis in endoscopy accompanied by elevated MEICS score (colon wall thickening, disturbed vascularization pattern, granular appearance of the colon wall, fibrin deposits, and loose stool, Figure 1B+C), shortening of the colon (Figure 1D), increased levels of myeloperoxidase in colon tissue (Figure 1E), and histological signs of severe colitis (erosion of the epithelium, infiltration of immune cells, edema in the sub-epithelium; figure 1F-I).

To test whether IMM-124E prevents colitis development, mice were treated with 100 mg/kg body weight or 500 mg/kg IMM-124E per day, starting two days before beginning of DSS application (preventive treatment). Administration of 100 mg/kg colostrum from non-immunized cows served as a treatment control. Another group received 500 mg/kg IMM-124E in a therapeutic setting, starting the gavages at day three of the DSS treatment. Of interest, all treatment schedules resulted in less severe weight loss in comparison to DSS only (Figure 1A), while colon length was not changed in any of the treatment groups (Figure 1D). However, other indicators of colitis severity showed clear differences between the treatment groups: for instance, MPO levels were only reduced in mice treated with 100 mg/kg IMM-124E, while 500 mg/kg IMM-124E (preventive or therapeutic) and colostrum administration did not affect MPO activity (Figure 1E). Preventive treatment with IMM-124E (100 mg/kg and 500 mg/kg) or with colostrum resulted in less severe colitis, as observed in mouse

endoscopy, while therapeutic treatment did not significantly reduce macroscopic signs of colitis (Figure 1B+C). Preventive 100 mg/kg IMM-124E treatment had the most pronounced effect, which was most overt in reduced levels of disturbed vascularization and granularity of the colon wall. Histological assessment of the terminal colon revealed that preventive treatment with 100 mg/kg IMM-124E significantly reduced epithelial damage and, albeit not statistically significant ( $p=0.06$ ), infiltration of immune cells into the submucosal layer, resulting in a reduction of total histology score (Figure 1F-I). Of note, none of the other treatments did result in a significant reduction of histological parameters of colitis. This clearly indicates that IMM-124E treatment prevents DSS-induced colitis, and that treatment with 100 mg/kg IMM-124E is most effective.

### **IMM-124E reduces pro-inflammatory cytokine production**

To understand the molecular mechanisms how IMM-124E reduced the extent of colitis in the acute DSS model, we next studied mRNA expression of pro- and anti-inflammatory cytokines in colon tissue from DSS-treated mice that received IMM-124E either preventive (100 mg/kg and 500 mg/kg) or therapeutically (500 mg/kg). Of note, DSS-treatment resulted in enhanced expression of *Il6*, *Il10*, *Ifng* and *Tnfa*. While preventive IMM-124E and colostrum administration inhibited the increase in *Il6* and *Il10* mRNA expression (Figures 2A+B), elevated *Ifng* and *Tnfa* expression was still present (Figure 2C+D). In line with increased mRNA expression upon DSS-administration, IL-6, IFN- $\gamma$  and TNF cytokine levels in the supernatant of colon explants from DSS-treated mice were also elevated (Figure 2E-H). Of interest, cytokine levels of IL-6, IFN- $\gamma$  and TNF were significantly reduced in the supernatant of colon explants from mice that received 100 mg/kg IMM-124E, while preventive



treatment with 500 mg/kg IMM-124E resulted in decreased IL-6 and IFN- $\gamma$  levels only and treatment with 100 mg/kg colostrum had no effect on the secretion of these cytokines (Figure 2E, G+H). IL-10 levels were not significantly affected by any of the treatments (Figure 2F).

### **IMM-124E ameliorates T cell mediated colitis.**

DSS-treatment results in erosion of the epithelial layer, and thus represents important features that contribute to colitis onset in IBD patients. However, it only partially reflects the involvement of immune cells in the pathogenesis of intestinal inflammation. In particular, the effect of pathogenic T cell responses is not reflected in this model<sup>31</sup>. Therefore, we next investigated the effect of IMM-124E treatment in the T cell transfer model of colitis, where naïve T cells are transferred into B- and T-cell deficient recipients. As expected, T cell transfer resulted in first signs of colitis after three weeks, characterized by onset of diarrhoea and macroscopic signs of colon inflammation in mouse endoscopy (Figure 3A). Upon onset of colitis, mice were randomized into groups with equal colitis severity and treated with 25 mg/kg, 100 mg/kg, or 500 mg/kg IMM-124E once per day. Since colostrum itself contains high levels of IgG and other factors that might act as confounders, administration of 100 mg/kg BSA or transfer of regulatory T cells served as controls. Although weight development did not differ significantly between the treatment groups (not shown), colonoscopy revealed that colitis was clearly reduced in mice receiving 100 mg/kg IMM-124E two weeks after the start of IMM-124E treatment (Figure 3B), an observation that was confirmed by assessment of spleen weight, colon length, and histology scores (Figure 3C-F).

### **IMM-124E affects immune cells in the inflamed colon.**

It has previously been shown that IMM-124E treatment results in enhanced levels of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in a TNBS-mediated colitis model as well as in NASH patients<sup>22, 23</sup>. To address whether IMM-124E treatment also affects CD4<sup>+</sup> T cells in the transfer colitis model, we performed flow cytometry on mesenteric lymph nodes (mLN) and colonic lamina propria lymphocytes (LPL). Flow cytometry revealed that administration of IMM-124E resulted in reduced levels of Th effector cells in mLN and LPL. In particular, proportions of CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> Th1, IL-17<sup>+</sup> Th17, and IL-13<sup>+</sup> Th2 cells were reduced (Figure 4A-C), while the proportion of FoxP3<sup>+</sup> regulatory T cells was enhanced in the colonic lamina propria and mLN of mice treated with 100 mg/kg IMM-124E (Figure 4D). While 25 mg/kg and 500 mg/kg IMM-124E treatment had some effect on Th cell subsets, this was clearly less pronounced. On the other hand, numbers of NK1.1<sup>+</sup> NK cells were very low and no significant effects were observed upon colitis induction or IMM-124E treatment (Figure 4E). Of note, all the beneficial effects observed upon IMM-124E treatment were absent in the control group receiving BSA.

### **IMM-124E prevents systemic LPS exposure upon colitis induction.**

IMM-124E contains high levels of anti-LPS IgG, and might therefore neutralize LPS translocation from the gut lumen into the systemic circulation. Although LPS itself is typically below detection limits, systemic exposure to LPS results in expression of LPS-binding protein (LBP), and LBP levels correlate with the extent of LPS exposure. Therefore, we next addressed whether LBP levels are increased in DSS colitis, and whether IMM-124E administration might affect LBP levels in the serum of DSS treated mice. In line with a severe barrier defect, DSS-treatment resulted in

358 significantly enhanced serum levels of LBP (Figure 5A). IMM-124E administration  
 359 significantly reduced serum levels of LBP. However, and in contrast to a better  
 360 response to 100 mg/kg IMM-124E with respect to colitis indicators, this effect was  
 361 not dose-dependent, i.e. 500 mg/kg IMM-124E administration was as effective as 100  
 362 mg/kg, while administration of colostrum did not affect serum LBP levels (Figure  
 363 5A). Nevertheless, levels of LBP moderately correlated with histological scores  
 364 (Figure 5B), indicating that systemic LPS translocation might be involved in  
 365 mediating disease in DSS-induced colitis. LBP serum levels were also increased in T  
 366 cell transfer colitis (Figure 5C), however to a lesser extent than in the DSS model.  
 367 Again, IMM-124E administration reduced LBP levels in the serum, but there was  
 368 only a weak correlation between LBP levels and total histology scores (Figure 5D).  
 369 To investigate whether there is direct presence of LPS in the mouse serum after colitis  
 370 induction, we next used HEKble-mTLR4 cells. These cells carry a reporter for  
 371 mouseTLR4 activation. When HEKble-mTLR4 cells were incubated with serum from  
 372 control mice, there was no TLR4 activation detectable. DSS-treatment in contrast  
 373 resulted in TLR4 activation, indicating presence of LPS in those mice. In line with  
 374 reduced LBP serum levels, TLR4 activation was lower in cells incubated with serum  
 375 from IMM-124E-treated mice. Again, the 100mg/kg IMM-124E was the most  
 376 effective dose (Figure 5E). To further investigate whether IMM-124E treatment  
 377 reduces LPS-induced signalling cascades in DSS-treated mice, we analysed activation  
 378 of NF- $\kappa$ B in colon lysates from mice with acute colitis with or without IMM-124E  
 379 treatment. We saw a strong induction of NF- $\kappa$ B p65 phosphorylation in DSS-treated  
 380 mice, which was clearly suppressed upon treatment with IMM-124E (Figure 5F). To  
 381 address whether IMM-124E treatment also results in reduced translocation of whole  
 382 bacteria, we measured levels of bacterial 16S rDNA in mesenteric lymph nodes from

our mice. However, we only observed 16S rDNA in very few mice, and did not observe any clear pattern (data not shown). Taken together, these data indicate that IMM-124E-treatment prevents DSS-induced systemic exposure to LPS and LPS-induced pro-inflammatory signalling.

#### **Therapeutic potential of IMM-124E is partially dependent on presence of IgG.**

To further investigate the mechanism of action of IMM-124E, we next performed an acute DSS colitis experiment using either the normal IMM-124E preparation, or IgG-depleted IMM-124E. In line with our findings that IMM-124E-treatment reduces exposure to LPS, which is likely due to high levels of anti-LPS antibody load, IgG depletion from IMM-124E partially abrogated the beneficial effect of IMM-124E (Figure 6).

## 398 Discussion

399 In this study, we demonstrated that administration of IMM-124E ameliorates colitis in  
400 two mechanistically different mouse models of intestinal inflammation, namely the  
401 DSS model of epithelial injury, and the immune-cell mediated T cell transfer colitis.  
402 IMM-124E administration significantly reduced DSS-mediated induction of pro-  
403 inflammatory cytokines in the intestine, including IL6 and TNF. In T cell transfer  
404 colitis, IMM-124E administration reduced levels of Th1, Th2, and Th17 cells, but  
405 promoted the induction of regulatory T cells. Further, IMM-124E significantly  
406 reduced systemic LPS exposure/presence of LPS in the serum.

407  
408 Of interest, the most effective dose of IMM-124E administration was 100mg/kg,  
409 which was even more effective than higher doses of IMM-124. Since the exact  
410 mechanism how Imm-124E works is still elusive, it is hard to tell why this might be  
411 the case. One possible explanation could be that high doses of IMM-124E might  
412 result in the formation of immune complexes of IgG with LPS, which could activate  
413 antigen-presenting cells in the intestine and thus promote pro-inflammatory effects.  
414 On the other hand, IMM-124E is a cow milk based product. Adult mice do not usually  
415 ingest milk and in combination with a barrier defect exposure to high levels of milk  
416 proteins might contribute to disease symptoms and thus suppress the beneficial effect  
417 of IMM-124E. However, further experiments would be required to understand the  
418 exact mechanism why IMM-124E is most effective at 100mg/kg.

419  
420 A recent study that investigated how IMM-124E might exert its anti-diarrheagenic  
421 effects, demonstrated that IMM-124E has direct anti-bacterial activity via inhibition  
422 of growth and migration of bacteria<sup>21</sup>. This indicates that also during colitis, the

beneficial effect of IMM-124E is possibly due to direct anti-bacterial properties, which might lead to decreased translocation of invading pathogens. However, we only observed 16S rDNA transcripts in mesenteric lymph nodes from very few DSS-treated mice. Additional experiments would be required to assess whether direct anti-microbial properties of IMM-124E play a role in protecting from colitis,.

It has previously been demonstrated that IMM-124E administration ameliorates insulin resistance and alleviates liver injury in a mouse model of NASH<sup>22</sup>, an effect associated with alteration in regulatory T cell and NKT cell proportions. In a small, open-label trial with 10 NASH patients, IMM-124E administration alleviated insulin resistance and improved lipid profile, which was accompanied by increased levels of regulatory T cells<sup>23</sup>. It has further been demonstrated that IMM-124E alleviates immune-mediated colitis in the TNBS model<sup>26</sup>. Also in this model, IMM-124E treatment resulted in elevated levels of regulatory T cells, and serum levels of IL-10 were significantly enhanced<sup>26</sup>. While we also found significantly elevated levels of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells in the transfer colitis model, we did not see an effect on NK cells, possibly because the abundance of these cells is rather low in mLN and the lamina propria, and their abundance might be generally altered in RAG2<sup>-/-</sup> recipients due to absence of T- B- and NKT cells.

In colitis, altered cytokine responses significantly contribute to disease development and exacerbation<sup>32</sup>. IL-10 is one of the most important anti-inflammatory cytokines for the maintenance of intestinal health<sup>33-35</sup>, and defects in the IL-10 signalling pathway predisposes to very early onset of IBD<sup>36, 37</sup>. While Ya'acov *et al* found increased levels of IL-10 in the serum of TNBS treated mice that received IMM-

124E<sup>26</sup>, we found that IMM-124E prevented DSS-induced induction of *IL10* mRNA expression, and there was no significant effect on IL-10 secretion. This indicates that in the DSS model, the effect of IMM-124E does not seem to be mediated via effects on the regulatory cytokine IL-10. In NASH patients treated with IMM-124E, an increase in IL-6 serum levels was observed<sup>23</sup>. However, in the DSS model, we observed an induction of IL-6 upon DSS-treatment, which was reduced in IMM-124E treated mice. This might be due to different modes of action, i.e. the role of IL-6 in NASH remains controversial, with studies showing that blocking of IL-6 promotes disease<sup>38</sup> and activation of IL-6 signalling alleviated disease in a NASH model<sup>39</sup>, whereas anti-IL6 treatment improved barrier function in DSS colitis<sup>40</sup> and ameliorated disease in T cell transfer colitis<sup>41</sup>

T helper cells play a major role in the development and progression of IBD<sup>42</sup>, and several studies demonstrated enhanced levels of IFN- $\gamma$ + Th1 and IL-17+ Th17 cells, while relative numbers of regulatory T cells are decreased (reviewed in<sup>43</sup>). IMM-124E administration corrected the altered balance in Th cells in the transfer colitis models, via reduction of exacerbated Th cell responses, and concomitant enhanced induction of regulatory T cells. This clearly demonstrates that IMM-124E administration reduces aberrant Th cell activation during colitis, and has the ability to promote anti-inflammatory responses.

NK cells and NKT cells are unique subsets of immune cells that produce large amounts of IFN- $\gamma$  and TNF upon activation. Further, they exert cytotoxic functions and lyse aberrant cells, including tumour cells<sup>44</sup>. Adar *et al* described increased levels of NK and NKT cells in IMM-124E treated ob/ob mice<sup>22</sup>. However, we could not

determine any difference in NK cell numbers in mesenteric lymph nodes or the lamina propria of IMM-124E treated mice. Of note, NKT cells are absent in RAG2<sup>-/-</sup> mice, and cannot be studied in this model. The differences in NK cell induction between our study and the study from Adar *et al* might further be explained by the fact that NK cells exert different effects depending on the disease context<sup>44</sup>. In experimental models of NASH, injection of NK cells alleviated disease via regulation of macrophage activation<sup>45, 46</sup>, while studies of NK cell numbers in IBD have yielded conflicting results<sup>47, 48</sup>.

Especially in the DSS colitis model, innate immune cells, including cells of the myeloid lineage, such as macrophages and granulocytes, are important drivers of disease<sup>31</sup> and there is evidence that these cells also play crucial roles in the onset of IBD in humans<sup>49, 50</sup>. While we did not address immune cells in detail in the DSS model, we measured MPO levels, which are a good indicator of myeloid cell infiltration and function<sup>51</sup>. In line with decreased levels of infiltrating immune cells into the inflamed colon of IMM-124E treated mice, MPO levels were also significantly decreased, indicating that in addition to prevent aberrant induction of Th cells, IMM-124E also prevents the accumulation of activated myeloid cells.

Summarized, our results demonstrate that IMM-124E administration significantly ameliorates experimental colitis and might be a promising novel therapeutic agent in IBD, either as a stand-alone treatment for maintaining remission in mild colitis cases or in combination with other treatments to increase therapeutic efficacy and prevent loss of therapeutic response.



497 **Conflict of interest**

498 This study was financed by Immuron Ltd. The sponsor had no role in the  
499 experimental design as well as in the interpretation, discussion and presentation of the  
500 results.

501

502 **Author contribution**

503 MRS, KA, KB, HM: performed experiments; MRS, IFW: data analysis; DRP, GL,  
504 GR, MS: intellectual discussion and critical scientific input; MS, GR, IFW: study  
505 design; all authors wrote, corrected and discussed the manuscript.

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## 655 **Figure Legends.**

656 **Figure 1. IMM-124E administration prevents acute DSS colitis.** Acute colitis was  
 657 induced in female C57Bl/6J mice via administration of 2.5% DSS in the drinking  
 658 water for seven days (day 2-9 of the experiment). Mice received 100 mg/kg or 500  
 659 mg/kg body weight IMM-124E per daily oral gavage starting at day 0 (preventive  
 660 treatment) or at day 4 (therapeutic treatment). Depicted are **A)** weight development,  
 661 **B)** representative pictures from mouse endoscopy and **C)** respective scoring according  
 662 to the of murine endoscopy index of colitis severity (MEICS) score, **D)** colon length,  
 663 **E)** myeloperoxidase (MPO) activity in the colon, **E)** representative pictures of H&E  
 664 stained terminal colon sections and scoring of **F)** epithelial damage, **G)** inflammation,  
 665 and **H)** summarized histological score. Asterisks denote significant differences  
 666 (\*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ ) A+C: n=6 per group, data are represented as  
 667 mean  $\pm$  SEM. D-H: each dot represents one individual mouse, horizontal bars stand  
 668 for mean, whiskers indicate SEM.

670 **Figure 2. IMM-124E prevents DSS-induced elevation of IL-6, IFN- $\gamma$  and TNF- $\alpha$ .**  
 671 Acute colitis was induced in female C57Bl/6J mice via administration of 2.5% DSS in  
 672 the drinking water for seven days (day 3-9 of the experiment). Mice received 100  
 673 mg/kg or 500 mg/kg body weight IMM-124E per daily oral gavage starting at day 1  
 674 (preventive treatment) or at day 5 (therapeutic treatment). **A-D):** mRNA expression  
 675 levels of the indicated genes in the terminal colon were analysed by RT-PCR and  
 676 normalized to *Gapdh* and the median of untreated controls. **E-H):** Colon pieces were  
 677 incubated in RPMI for 24 h and levels of the indicated cytokines in the supernatant  
 678 analysed by ELISA. Each dot represents an individual mouse, horizontal bars indicate

mean, and whiskers indicate SEM. Asterisks denote significant differences (\*= $p<0.05$ , \*\*= $p<0.01$ ).

**Figure 3. IMM-124E ameliorates T-cell transfer colitis.** Colitis was induced in RAG2<sup>-/-</sup> mice via transfer of  $0.5 \times 10^6$  naïve T cells. Control mice received no T cells or  $0.5 \times 10^6$  naïve T cells +  $0.5 \times 10^6$  regulatory T cells. At day 24, colonoscopy was performed to verify colitis induction, and mice were distributed into groups with equal colitis severity. Starting at day 24, mice received 25 mg/kg, 100 mg/kg or 500 mg/kg IMM-124E, or 100 mg/kg BSA per daily oral gavage until the end of the experiment 4 weeks later. Depicted are **A)** representative pictures from colonoscopy on day 24, **B)** representative pictures and scoring according to the murine endoscopic index of colitis severity (MEICS) score from colonoscopy at the end of the experiment, **C)** spleen weight, **D)** colon length, **E)** histological scoring and **F)** representative pictures of H&E stained sections of the terminal colon. Each dot represents an individual mouse horizontal bars indicate mean, and whiskers indicate SEM.  $n=7$  for each group, except control without T cells ( $n=8$ ) and BSA ( $n=5$ ). Asterisks denote significant differences (\*= $p<0.05$ , \*\*= $p<0.01$ ).

**Figure 4. Altered immune cell subsets upon treatment with IMM-124E.** Colitis was induced in RAG2<sup>-/-</sup> mice via transfer of  $0.5 \times 10^6$  naïve T cells. Control mice received no T cells or  $0.5 \times 10^6$  naïve T cells +  $0.5 \times 10^6$  regulatory T cells. At day 24, colonoscopy was performed to verify colitis induction, and mice were distributed into groups with equal colitis severity. Starting at day 24, mice received 25 mg/kg, 100 mg/kg or 500 mg/kg IMM-124E, or 100 mg/kg BSA per daily oral gavage until the end of the experiment 4 weeks later. Immune cells from the mesenteric lymph nodes

(mLN) or the lamina propria (LPL) were analysed by flow cytometry for **A-D**) T cells expressing the indicated cytokines and **E**) proportion of NK cells. Each dot represents an individual mouse, horizontal bars indicate mean, and whiskers indicate SEM. Asterisks denote significant differences (\*= $p<0.05$ , \*\*= $p<0.01$ ).

**Figure 5. IMM-124E prevents systemic exposure to LPS. A+B)** Acute colitis was induced in female B57BL/6 mice via administration of 2.5% DSS in the drinking water for seven days (day 3-9 of the experiment). Mice received 100 mg/kg or 500 mg/kg body weight IMM-124E per daily oral gavage starting at day 1 (preventive treatment) or at day 5 (therapeutic treatment). Serum was collected at the end of the experiment and analysed for the concentration of LPS-binding protein (LBP) by ELISA (A) and LBP levels were correlated to total histology scores from the same mice (B).  $n=6$  per group. **C+D)** Colitis was induced in RAG2<sup>-/-</sup> mice via transfer of  $0.5 \times 10^6$  naïve T cells. Control mice received no T cells or  $0.5 \times 10^6$  naïve T cells +  $0.5 \times 10^6$  regulatory T cells. At day 24, colonoscopy was performed to verify colitis induction, and mice were distributed into groups with equal colitis severity. Starting at day 24, mice received 25 mg/kg, 100 mg/kg or 500 mg/kg IMM-124E, or 100 mg/kg BSA per daily oral gavage until the end of the experiment 4 weeks later. Serum was collected at the end of the experiment and analysed for the concentration of LPS-binding protein (LBP) by ELISA (C) and LBP values correlated with total histology scores from the same mice (D).  $n=7$  in each group, except no T cells control ( $n=8$ ) and BSA treated ( $n=5$ ) group. **E)** Serum from mice in A (acute DSS colitis) and C (T cells transfer colitis) was incubated with HEKblue-mTLR4 reporter cells for 48h to determine presence of LPS. SEAP activity levels were measured at 620nm and correspond to the amount of LPS present in the serum. **F)** Representative pictures and



respective densitometric analysis of colon lysates from mice treated as in A (acute colitis). n=3 for each treatment condition. Each dot represents an individual mouse, horizontal bars indicate mean, and whiskers indicate SEM. Asterisks denote significant differences (\*= $p<0.05$ , \*\*= $p<0.01$ ). In B+D the line indicates the best-fit.

**Figure 6. Depletion of IgG from IMM-124E partially abrogates its beneficial effect.** Acute colitis was induced in female C57Bl/6J mice via administration of 2.5% DSS in the drinking water for seven days (day 2-9 of the experiment). Mice received 100 mg/kg body weight normal IMM-124E, or IgG-depleted IMM-124E per daily oral gavage starting at day 0. Depicted are **A)** weight development, **B)** representative pictures from mouse endoscopy and **C)** respective scoring according to the of murine endoscopy index of colitis severity (MEICS) score, **D)** colon length, **E)** representative pictures of H&E stained terminal colon sections, and **F)** histological scoring. Asterisks denote significant differences (\*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ ) n=5 per group, data are represented as mean  $\pm$  SEM, and each dot represents one individual mouse, horizontal bars stand for mean, whiskers indicate SEM.